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14. ABSTRACT

The carcinogenic effects of low dose radiation are not clear. Moreover, it is not known if exposure to low dose radiation increases cancer risk in offspring of irradiated parents. Radiation is known to damage DNA and cause mutations. Cancer arises in part through mutations in oncogenes and tumor suppressor genes. However, epigenetics is also known to play an important and perhaps central role in cancer induction. Epigenetics is here defined as a heritable change in phenotype without an underlying mutational event. Epigenetic alterations may be induced early in development as a result of diet or environmental exposure. These alterations can persist into adulthood, and may increase the risk of developing diseases such as cancer later in life. Radiation has been shown to cause epigenetic effects in mammalian cells and in mice, although the role of these in human cancer is entirely unknown. Here we hypothesize that the carcinogenic effect of radiation may in part be due to epigenetic alterations. In this study we will determine if early exposure to radiation leads to a change in DNA methylation in adult tissues and in ensuing unexposed generations, and if these changes correlate with increased cancer susceptibility. We also expect to identify a "radiation exposure signature" which when validated, could be used clinically as a biomarker of radiation exposure. These experiments will also open up new avenues of investigation into the role of epigenetics in cancer caused by radiation and other environmental carcinogens.

15. SUBJECT TERMS
Epigenetic biomarkers, transgenerational, lung cancer

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Transgenerational Radiation Epigenetics

Introduction

This study is designed to determine whether ionizing radiation results in changes to DNA methylation and if these changes are heritable, epigenetic changes. We hypothesize that exposure to low dose irradiation in utero will result in alterations to the pattern of DNA methylation in normal tissue and that at least some of these changes will then be passed on to future generations. We also hypothesize that these changes in methylation may lead to increased rates of cancer. We are using a mouse model of in utero exposure and are focusing specifically on lung cancer.

Body

We used a Balb/c mouse model that spontaneously develops lung cancer at an incidence of ~30%. We exposed pregnant mice at E15 to 0.5Gy (50rads) of whole body irradiation. The resulting offspring (F1) were used in primary studies, with tissue collected at 10 weeks of age or allowed to age up to two years, collecting tissue when they show signs of tumor burden. We originally proposed sacrificing mice for tumor analysis at 40 weeks of age, however our preliminary analysis indicated this was too early so we extended the tumor study endpoint to 100 weeks of age.

F1 offspring were also bred to generate F2 mice. Mice from the F2 generation were then bred to generate F3 mice. Unirradiated controls were set up for the 10 week experiment, long term tumor experiment, and transgenerational study.

We originally proposed to collect only lung tissue for methylation analysis. However, we expanded this and collected normal tissue from lung, liver, kidney, spleen, teste or ovary, heart and plasma for analysis. A piece of each tissue was taken for fixation in NBF and the rest of the tissue was snap frozen. Samples (except for plasma) for freezing were put into beem capsules and frozen in liquid nitrogen. Blood for plasma was collected and put into EDTA tubes, allowed to sit then centrifuged to remove blood cells. The supernatant (plasma) was then moved into a cryotube and snap frozen. All frozen tissues are currently stored in a -80 freezer.

Although the focus of this study was originally on the male paternal line, we have also collected tissues from the female line and experimental animals. We will use these samples both for comparison to the males and for possible future studies. This effectively doubled the number of experimental animals.

In addition we have also collected samples from the breeders.

Thus we expanded the number of tissues collected, doubled the endpoint for tumor analysis and doubled the number of mice in the transgenerational study. This has generated an invaluable tissue bank for subsequent comprehensive genomic and transcriptomic profiling.

Specific Aims

Specific Aim 1: Identify and validate epigenetic biomarkers of low dose radiation in normal lung tissue. All the samples for Aim 1 have been collected. In an initial study we examined DNA from the lung samples collected at 10 weeks. Six irradiated and 6 unirradiated samples were analyzed using the Qiagen EpiTect Methy II PCR Array Mouse Lung Cancer Complete Panel (EAMM-8040Z). We found that in both the irradiated group and non-irradiated group seven genes in this panel were hypermethylated, three over 50% including Gata6, Stratifin and Tnfrsf25. It is interesting to note that Gata6 is important for lung epithelial development and along with Stratifin is involved in epithelial cell differentiation. This could be indicative of why these mice develop lung tumors. We are currently isolating RNA to look at mRNA levels by qPCR of specific

cancer related genes. We are using a 384 plate format from Bio-Rad. In order to facilitate comparisons across all samples and generations, we plan to perform all the analyses in one batch. As we wait for the final samples from Aim 3 we are trouble shooting the technologies, in particular whole genome methylation analysis.

Specific Aim 2: Identify epigenetic alterations in radiation-induced lung tumors. We have just finished collecting tissues from the long-term male and female mice which as stated above was extended to a two year endpoint. There was no significant difference in survival between the irradiated versus the non-irradiated male mice. However in the female mice there was a significant (p=0.04) difference between the two groups (Fig 1).

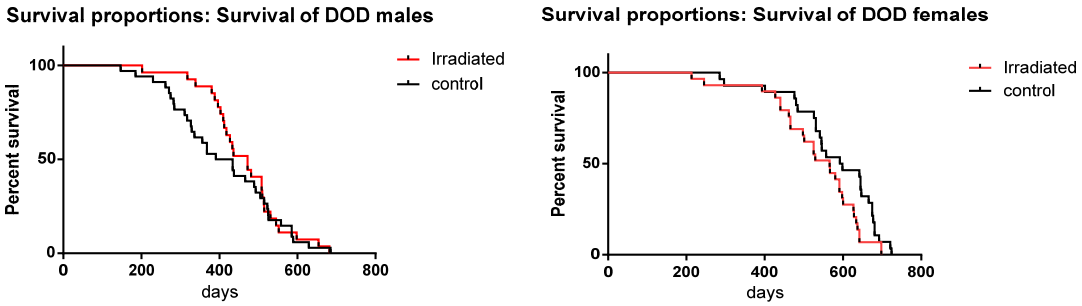

Figure 1. Survival curves of irradiated male and female F1 mice.

In both the males and females we found increases in lung tumor incidence in irradiated vs. non-irradiated mice (Fig 2). In the male mice the increase was modest, while the incidence of tumors in females increased by nearly 50% over the non-irradiated females (17.2% to 29.0%).

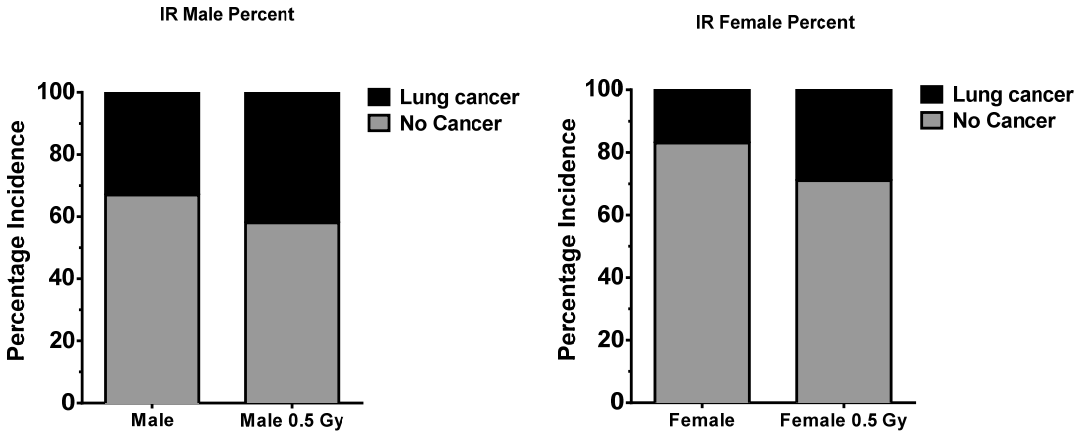

Figure 2. Percent of lung tumors in irradiated vs. non-irradiated mice.

In both the irradiated and non-irradiated groups the incidence of tumors was higher in the male mice (Fig 3). However the difference between male vs. female was less in the irradiated group.

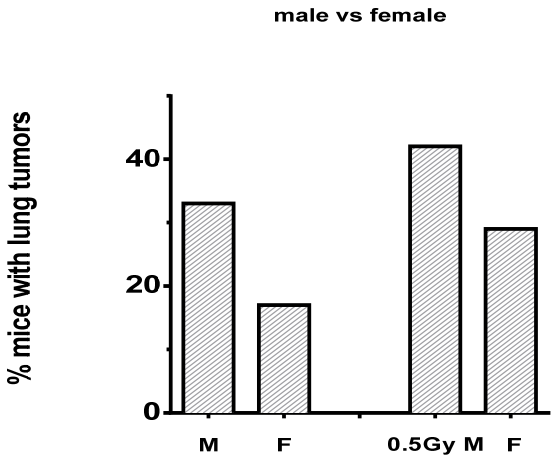

Figure 3. Percent of males (M) vs. female (F) mice with lung tumors in control and irradiated mice.

In the non-irradiated male mice the average size of the lung tumors was 3.9 ± 2.5 mm compared to 4.4 ± 4.7mm in the irradiated mice. The largest tumor (20mm) was found in the irradiated group. In the non-irradiated females the average size of the tumors was 2.1±1.7mm compared to 4.6±3.2 mm in the irradiated group. The largest tumor was also found in the irradiated cohort (12mm). We are currently analyzing histopathology of all lung tumors to determine if there are differences in pathology that associate with radiation exposure (Fig 4). The epithelial to mesenchymal transition (EMT) status of the tumors will be examined using E-cadherin and Vimentin straining We may also use Histone H3, H2A.X, Ki-67 and Caspase 3 staining to determine growth and DNA damage status.

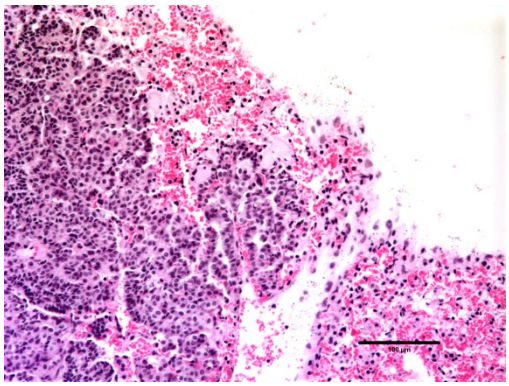

Figure 4 Invading edge of lung tumor from irradiated male mouse.

Specific Aim 3: Identify epigenetic changes in unexposed mice derived from irradiated parents or grandparents. To date we have collected tissues from all the 10 week old mice, both irradiated and control groups and from all F2 mice. We have just finished collecting tissues from the F3 maternal generation and over the next 6 months will finish collecting the F3 male generation samples. All mice were weighed at necropsy. We collected not only lung for the experiments outlined in this grant expanded this list to include liver, kidney, spleen, testes/ovary, heart and plasma for possible future studies. At 10 weeks of age, irradiated mice of both sexes showed a reduced median body weight (Fig.5). In the paternal line, the weights of the males were at control levels by the F2 generation. In the maternal line, the body weights of the females remained low in the F2 generation and recovered by the F3 generation.

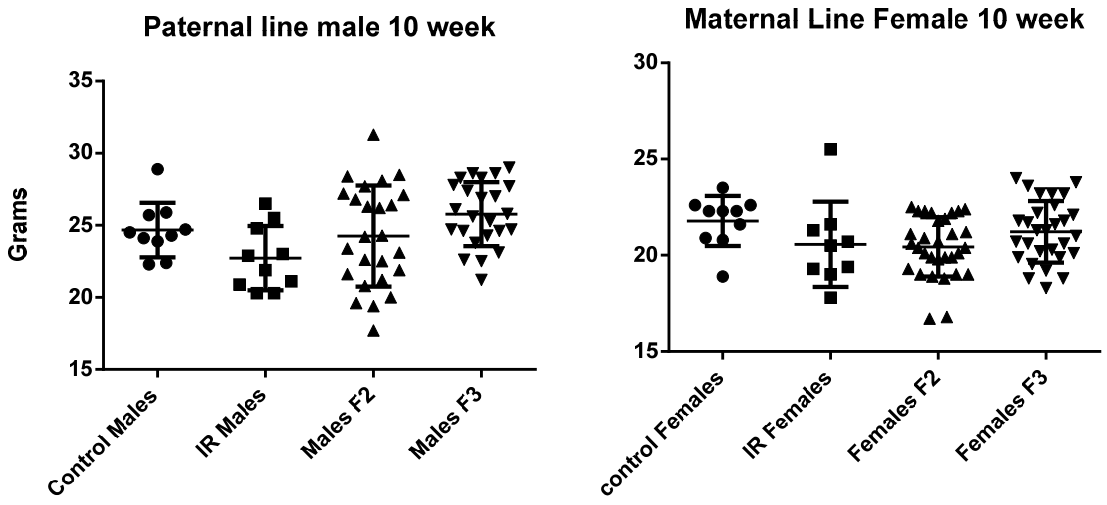

Figure 5. Transgenerational body weights of irradiated and non-irradiated cohorts.

We have been granted a one year extension to complete the project. Once all tissues are collected we will proceed with gene expression and methylation analyses.

Key Research Accomplishments

- Collection of all samples for Aim 1
- Initial analyses of samples from Aim 1
- Collection of all samples from Aim 2
- Initial analyses of results from Aim 2
- Collection of all samples for paternal line and most samples for the maternal line for Aim 3

Reportable Outcomes

We have banked tissues from both male and female mice including liver, lung , kidney, and spleen for future studies.

We have obtained evidence that in utero irradiation leads to reduced body weight in young adult mice and that this is transmitted through the maternal line in a subsequent generation.

We have evidence that in utero irradiation leads to increased incidence of lung tumors in susceptible mice.

Conclusions

In utero radiation increases lung cancer and impairs body weight gain.

In early 2012 our mouse room was put on quarantine due to pinworm contamination that arose from another investigator's mice. Although our mice never tested positive all mice were put on chow containing Fenbendazole from May 14 to Aug 1, 2012. During this time no live mice were allowed to leave the room. We do not anticipate any adverse outcomes from the diet but as no studies have been done on methylation changes due to Fenbendazole we cannot completely rule it out. Since both controls and irradiated groups were on the diet this should negate any effect on our results.

References & Appendices: None.